

PROOF CERTIFICATE OF MAILING BY "EXPRESS MAIL"
"Express Mail" Mailing Label No. **TE 06913253**
DEC 17 1993 Date of Deposit **DECEMBER 17, 1993**
I hereby certify that this paper or fee is being deposited
with the United States Postal Service "Express Mail Pos
Office to Addressee" service under 37 CFR 1.10 on the date
indicated above and is addressed to the Commissioner of
Patents and Trademarks, Washington D.C. 20231

962001300

ENT 169293

A/ NO fee

-1-

WILLIAM J WYTENBURG

(Typed or Printed Name of Person Mailing Paper or Fee)

William J Wytenburg
(Signature of Person Mailing Paper or Fee)

A METHOD OF PREVENTING DEPLETION OF
NON-AUTOLOGOUS HEMATOPOIETIC CELLS
AND ANIMAL MODEL SYSTEMS FOR USE THEREOF

10 Technical Field

The invention is in the field of hematopoietic cell reconstitution.

Background Art

15 Animals including man tend to deplete rapidly non-autologous peripheral hematopoietic cells. This is true even if the animals are immunocompromised. This phenomenon has hindered the use of allogeneic cell transfusions in immunocompromised humans. Animal model
20 systems have been proposed to study the human hematopoietic and immunologic system. However, they are not adequately reflective of the full complement of the immune system due to the lack or decreased amount of xenogeneic peripheral hematopoietic cells.

25 The most frequently used animal models of the human immune system utilize severe combined immune deficiency (SCID) mice that have been injected with human peripheral blood cells or bone marrow cells; or that have been humanized by co-implantation of human fetal thymus
30 and liver (Thy/Liv) or by implantation of human fetal bone marrow fragments. Mosier et al. (1988) Nature, 335:256; Kamel-Reid and Dick (1988) Science, 242:1706; McCune et al. (1988) Science, 241:1632; Namikawa et al. (1990) J. Exp. Med., 172:1055; Kyoizumi et al. (1992)
35 Blood, 79:1704; Kyoizumi (1993) Blood, 81:1497; and

-2-

- Namikawa et al. (1993) Blood, 82:2526. Injection of human bone marrow followed by a regimen of cytokine injections has been shown to allow substantial prolongation of survival of human myeloid progenitors.
- 5 Lapidot et al. (1992) Science, 255:1137. The SCID-hu Thy/Liv grafts produce phenotypically normal human T cells in the periphery for prolonged periods, allowing mechanisms of tolerance induction and T cell repertoire to be studied. Krowka et al. (1991) J. Immunol.,
- 10 146:3751; Vandekerckhove et al. (1991) J. Immunol., 146:4173; Vandekerckhove et al. (1992) J. Exp. Med., 175:1033; Vandekerckhove et al. (1992) J. Exp. Med., 176:1619; and Baccala et al. (1993) J. Exp. Med., 177:1481.
- 15 SCID-hu mice are also valuable for the study of human hematopoietic stem cell development and human immunodeficiency virus (HIV) pathogenesis. Peault et al. (1991) J. Exp. Med., 174:1283; Baum et al. (1992) Proc. Natl. Acad. Sci. USA, 89:2804; McCune et al. (1990)
- 20 Science, 247:564; Bonyhadi et al. (1993) Nature, 363:728; Aldrovandi et al. (1993) Nature, 363:732; and copending co-owned United States patent application serial no. 07/882,937.
- The potential use of SCID-hu mice is limited,
- 25 however, by the low engraftment of human cells. The current models would be greatly improved by providing a means to allow human hematopoietic cells to survive and circulate freely in the periphery of immunocompromised mice. Moreover, methods useful in increasing engraftment
- 30 of human cells in SCID-hu mice have direct application in a variety of human disorders.
- The mononuclear phagocyte systems of the spleen and liver are important sites of phagocytosis of both opsonized and non-opsonized particles as well as in the
- 35 early phases of bacterial infection. Lockwood (1983)

-3-

Clin. Hematol. 12:449. Specific elimination of macrophages in the spleen and liver can be effected by injection of liposome-encapsulated dichloromethylene diphosphonate (Cl_2MDP). Van Rooijen et al. (1984) Cell Tissue Res., 238:355; and Van Rooijen and Claassen, *In vivo Elimination of Macrophages in Spleen and Liver, Using Liposome-Encapsulated Drugs: Methods and Applications* (John Wiley and Sons, Chichester, 1988). Injection of free Cl_2MDP does not result in significant macrophage depletion because small, charged molecules like Cl_2MDP are not subject to endocytosis by macrophages as liposomes are. Gregoriadis et al., *Targeting of Drugs* (Plenum Press, New York, 1982). Nor does the injection of liposome-encapsulated phosphate-buffered saline (PBS) reduce macrophage levels as neither the liposomes nor the PBS are toxic to the macrophage. However, a single intravenous injection of liposome-encapsulated Cl_2MDP results in the disappearance of splenic red pulp and marginal zone macrophages with recovery of these subpopulations occurring 1-2 weeks and greater than one month respectively. Van Rooijen et al. (1989) J. Leuk. Biol., 45:97. The Cl_2MDP -induced macrophage depletion can be sustained for prolonged periods (at least one month) with sequential intravenous injections. Kraal et al. (1993) Int. Arch. Allergy Immunol., 100:115.

It has now been found that the macrophages in the mononuclear phagocytic system play an important role in clearance of non-autologous hematopoietic cells ~~in~~ and elimination of endogenous macrophages results in the ability of non-autologous hematopoietic cells to circulate and survive in the periphery of host animals.

Summary of the Invention

Methods are provided for preventing depletion of peripheral non-autologous hematopoietic cells in

-4-

animals including humans by substantially ablating the endogenous macrophage population. Animal models of peripheral non-autologous hematopoietic cells and the full complement of the human hematopoietic system are provided.

Brief Description of the Drawings

Figure 1 depicts flow cytometric analyses of peripheral blood, spleen and bone marrow of individual SCID mice either non-treated (control) or treated with liposome-encapsulated Cl_2 MDP and injected with human Ficoll-purified peripheral blood leukocytes (Treatment 2) as described in Example 6 and Table 1.

Figure 2 is a summary of flow cytometric data of human cell content in peripheral blood, spleen and bone marrow and of splenic immunocytochemistry. Evaluation for CD45 staining is as follows: (-) no CD45⁺ cells visible; (+/-) few CD45⁺ cells in white pulp areas or not all white pulp areas populated; (+) clear presence of CD45⁺ cells in white pulp areas; and (++) many CD45⁺ cells in white pulp areas.

Figure 3 depicts the percent of human cell content in the peripheral blood of SCID-hu Thy/Liv mice over time.

Figure 4 depicts immunohistochemical and flow cytometric evaluation of macrophage depletion and human cell infiltration in liposome-encapsulated Cl_2 MDP treated SCID-hu Thy/Liv mice. Figure 4A depicts cryostat sections of SCID-hu Thy/Liv mice treated with PBS (a and b) or liposome-encapsulated Cl_2 MDP (c and d) and analyzed for acid phosphatase (a and c) or stained with antibodies specific for CD45 (b and d). The following abbreviations are used: wp, white pulp; and rp, red pulp of the spleen. Figure 4B depicts flow cytometry analyses of splenic cells from mouse 7635-3. Panel A depicts forward scatter

-5-

versus side scatter showing the gated region (R2).

Panel B depicts the isotype control profile of IgG1-FITC and IgG2a-TC staining. Panel C depicts the profile of cells stained with W6/32-FITC and CD45-TC stained cells.

5 Panel D depicts the histogram of CD45 staining (solid line) and isotype control (dashed line) with gates set for CD45⁺ cells (RI). Panel E depicts the histogram of CD8⁺ cells after gating on region R1 (panel D) and region R2 (panel A). The percentage of positive cells is
10 indicated in the top right corner. Panel F depicts the histograms of CD4⁺ cells after gating on R1 and R2.

Figure 5 is 3 graphs depicting the level of human hematopoietic cells in the peripheral blood of SCID-hu Thy/Liv mice. Figure 5A depicts the initial
15 transient increase of human hematopoietic cells after the Thy/Liv transplant. Figure 5B depicts the decrease in human hematopoietic cells after the human grafts are removed. Figure 5C depicts the transient increase of human cells upon removal of the spleen from SCID-hu
20 Thy/Liv mice.

Modes of Carrying Out the Invention

The results described herein indicate that macrophages in the mononuclear phagocytic system play an
25 important role in the clearance of hematopoietic cells in non-autologous animals, and the elimination of macrophages results in the ability of non-autologous hematopoietic cells to circulate and survive in the periphery. Human hematopoietic cells were completely
30 undetectable in peripheral blood, spleen and bone marrow of normal SCID mice when 5×10^6 human peripheral blood lymphocytes (PBLs) were injected intravenously (i.v.) and mice were analyzed 72 hours later for cells positive for human HLA class I and CD45 by FACS. In contrast, human
35 hematopoietic cells were readily detected in mice pre-

-6-

treated with liposome-encapsulated Cl_2MDP 72 hours after injection of human PBLs. In addition, a single injection of liposome-encapsulated Cl_2MDP into SCID-hu Thy/Liv mice resulted in an increase of human hematopoietic cells circulating in mouse peripheral blood for at least 2 weeks after the injection.

Histochemical analyses of splenic tissue of SCID mice treated with liposome-encapsulated Cl_2MDP demonstrated a correlation between elimination of acid phosphatase positive cells in the red pulp and marginal zones of the spleen and accumulation of CD45^+ cells in the white pulp. Intravenous treatment with liposome-encapsulated Cl_2MDP eliminates phagocytic cells in organs with open circulation, such as the spleen and liver, (Van Rooijen et al. 1984) but does not eliminate splenic white pulp macrophages in normal mice or in SCID mice as described here, presumably since the microcirculation in the white pulp region of the spleen does not allow immediate access to liposomes entering the spleen.

The invention provides methods of preventing depletion of non-autologous hematopoietic cells in animals including man. Non-autologous hematopoietic cells include all hematopoietic cells not produced by the animal itself (the host animal). Hematopoietic cells are those cells derived from hematopoietic stem cells and include, but are not limited to, B cells, T cells, natural killer cells, dendritic cells, macrophages, monocytes, mast cells, granulocytes and megakaryocytes. Non-autologous cells include both allogeneic and xenogeneic cells. The non-autologous cells have now been found to remain in the periphery of the host animal for a longer duration than the times obtained with previous methods. The periphery of the host animal includes, but is not limited to, blood circulation, thymus, tissues, bone marrow and secondary lymphoid tissues. The methods

-7-

involve decreasing the endogenous macrophages of the animal to a level effective to substantially prevent depletion of the non-autologous cells. The non-autologous hematopoietic cells may be directly injected
5 into the animal or may be produced in the animal by engrafted hematopoietic tissue.

Depletion of endogenous macrophages can be effected by any method known in the art, including, but not limited to, the transgenic elimination or
10 inactivation of macrophages, treatment with L-leucine methyl ester, and the administration of colloidal carbon to the reticuloendothelial system. Preferably, the animals are administered an agent or drug that specifically eliminates macrophages. More preferably,
15 the agent is liposome-encapsulated Cl_2 MDP. Alternatively, the macrophages are depleted genetically such as by producing an animal that does not produce or produces diminished levels of macrophages, or functionally inactive macrophages.

20 The methods are also suitable for use in immunocompromised animals to prolong survival of non-autologous hematopoietic cells. Suitable immunocompromised animals include, but are not limited to, humans, SCID mice, SCID-hu mice, CID horse, and
25 transgenic immunodeficient mice. Suitable immunodeficient mice include, but are not limited to, Class I, Class II MHC⁻, Bcl-2 proto-oncogene deficient mice and RAG deficient mice. RAG-1 or RAG-2 deficient mice lack the VDJ-recombinase activator genes.
30 Immunocompromised humans include, but are not limited to, those infected with HIV, those undergoing cellular ablative therapy including, but not limited to, radiation and chemotherapy, and humans suffering from SCID.

Preferably, the method is used in SCID mice and
35 the non-autologous hematopoietic cells are human. More

-8-

preferably, the SCID mice have been engrafted with at least one human hematopoietic tissue which tissue produces the peripheral hematopoietic cells. Suitable tissue for engraftment includes but is not limited to

5 thymus, liver, bone marrow, spleen and lymph nodes, and combinations thereof. Alternatively, the SCID mice may be injected directly with human hematopoietic cells, in conjunction with decreasing the endogenous macrophages.

In conjunction with, means that the endogenous macrophages
 10 *P* can be depleted before, *during* or after inducing expression of or directly injecting non-autologous hematopoietic cells.

The method is also suitable for use in humans, particularly those infected with HIV who lack certain

15 subsets of PBLs such as CD4⁺ lymphocytes. In these persons, an effective amount of human hematopoietic blood cells can be administered to the patient in conjunction with decreasing the endogenous macrophages. The hematopoietic blood cells are preferably hemato lymphoid,

20 more preferably T cells and most preferably, CD4⁺ lymphocytes. These cells may be injected directly into the bloodstream, for instance by intravenous injection, in conjunction with decreasing the endogenous macrophages. In addition the method can be used to

25 reduce endogenous macrophages in HIV patients receiving bone marrow transplantation therapy to increase the survivorship of the engrafted tissue.

The invention further encompasses the reduction of macrophages as a means of improving engraftment

30 efficiency for allogeneic and xenogeneic stem cell transplantation. Improving engraftment efficiency for transplantation of a population of non-autologous hematopoietic stem cells in a host animal includes the steps of ablating, in whole or in part, the endogenous

35 stem cell population of the host animal and transplanting

-9-

the stem cells into the patient in conjunction with decreasing endogenous macrophages in the host animal. Methods of obtaining populations of hematopoietic stem cells are known in the art as are methods of performing stem cell transplantations.

The non-autologous hematopoietic cells may be derived from any source. In the case of animal model systems, it is preferred that these cells are of human origin so as to more clearly define the human hematopoietic system. In the case of immunocompromised humans, it is preferred to use human cells, preferably those that are as closely histocompatibility matched to the patient as possible.

The non-autologous hematopoietic cells may contain the full complement of peripheral blood elements (PBE) or may comprise one or more subsets thereof. Normally, PBEs include, but are not limited to, red blood cells, lymphocytes, monocytes, and granulocytes. Any one of these subsets or a constituent thereof may also be used, such as discussed above for treatment of HIV-infected persons. Prior to introduction into the host animal, the hematopoietic cells can be separated into their different types by a variety of methods known in the art. These methods include, but are not limited to, flow-cytometry, immunoaffinity with antibodies to hematopoietic cells (chromatography and panning), counterflow centrifugation, separation by density gradients (like ficoll) and elutriation.

Typically, the non-autologous hematopoietic cells are introduced by intravenous injection. In the mouse, such injections are generally into the tail vein; in humans intravenous administration is by methods known in the art. If the cells have been separated, they should be resuspended in a suitable amount of a physiologically acceptable buffer. Such buffers are

-10-

known in the art and are suitably, sterile, non-pyrogenic and isotonic.

Decreasing the number of endogenous macrophages can be done by any method known in the art. Preferably, the macrophages are decreased by administration of an agent which selectively kills macrophages. For instance, see, Van Rooijen and Claasen (1988). More preferably, Cl_2MDP is administered in a manner whereby it is taken up by macrophages but not other cell types. Typically, the Cl_2MDP is encapsulated in liposomes. Methods of making liposome drug delivery vehicles are known in the art and are not described in detail herein. Such methods include, but are not limited to, the method described briefly in Example 6. Any method of forming liposomes that allows encapsulation of an effective amount of the agent is suitable for use in the present invention.

The effective amount of the agent is that which decreases the level of endogenous macrophages to a level effective to substantially prevent depletion of the non-autologous hematopoietic cells. In the case of liposome-encapsulated Cl_2MDP , the effective concentration in mice (both wild type and SCID) is in the range of 0.005 to 0.010 ml of liposomes (containing 23.5 mg/ml lipid per 10 to 15 mM Cl_2MDP) per gram of mouse weight. While extrapolating to humans is not directly proportional, typically, the effective range would be 5 to 10 ml of these liposomes per kg of human weight. Other macrophage-toxic substances will have different effective amounts but a determination of the effective amount is within the skill of one in the art. While the determination of an effective amount of a particular agent will be empirical, the effective amount can be determined empirically by monitoring survival of non-autologous hematopoietic cells in the periphery of the host animal. Preferably, the effective dosages are first

-11-

determined in mice and extrapolated to humans for subsequent preclinical testing.

Substantially preventing depletion of non-autologous hematopoietic cells indicates that for at least several days, and preferably up to several weeks, the cells are found in the peripheral blood of the animal. Preferably 1 % of the cells remain in the periphery after several days. More preferably 5 % of the cells remain in the periphery after several days. Most preferably 10 % of the cells remain in the periphery after several days.

Measurement of the number of non-autologous hematopoietic cells remaining in the periphery in order to determine the effectiveness of the agent can be accomplished by any method known in the art. Such methods must be merely able to distinguish between autologous and non-autologous cells. A variety of such markers are known in the art. In the case of xenogeneic cells, species specific cell surface markers including, but not limited to, histocompatibility markers can be used. In the case of allogeneic cells, markers including, but not limited to, histocompatibility antigens can be used.

The endogenous macrophages found in the red pulp area of the spleen appear to be responsible for depletion of the non-autologous hematopoietic cells. Therefore the level of these macrophages is preferentially decreased. Nonetheless, the invention is not limited to any particular mechanism of action of the agent; thus agents which kill all macrophages or at least one subset thereof are suitable for use in the present invention provided they substantially prevent depletion of non-autologous hematopoietic cells.

The invention further provides methods of treating animals including humans by administering non-

-12-

autologous hematopoietic cells and decreasing endogenous macrophages to a level sufficient to prevent substantial depletion of the non-autologous cells. Such treatment is particularly effective for immunocompromised patients as discussed above. The methods of administration are as discussed above.

The invention further provides non-human animal model systems of non-autologous hematopoietic systems. These animals comprise engrafted human hematopoietic tissue and have a decreased level of macrophages sufficient to prevent substantial depletion of human hematopoietic cells. Preferably, the animals are immunocompromised and include, but are not limited to, SCID-hu and SCID-hu Thy/Liv mice. The methods of engraftment and depletion of endogenous macrophages are as discussed above and in the examples presented herein.

The ability to enhance and prolong circulation of human hematopoietic cells in the SCID mouse with prolonged Cl_2 MDP-liposome treatment increases the potential for study of human hemopoietic, immunologic and disease processes *in vivo*. Applications include, but are not limited to, vaccine development, immunologic reactions to tumors and the development of human hybridomas.

Prolonged circulation of human hematopoietic cells in the periphery provides a means for human hematopoietic cell function to be studied *in vivo* such as homing, engraftment, and tumor or allogeneic rejection of foreign tissues which were previously not feasible, or more difficult. Examples include, but are not limited to, the pathogenesis of HIV where the kinetics of infection of a few circulating infected cells may be followed in the SCID-hu Thy/Liv HIV model. Maintenance of human bone marrow or circulating PBEs for longer periods should improve engraftment potential of these

-13-

populations, and decrease the cell dose required for engraftment making it possible to study development of defined human hemopoietic cell subtypes such as purified stem cells. Finally, the ability of increased numbers of human cells to circulate may allow propagation of cells from tumors or proliferative disorders such as acute or chronic myeloid leukemia which have been difficult to maintain in mice.

The following examples are meant to illustrate
10 but not limit the invention.

EXAMPLES

Example 1

Mice

CB-17 scid/scid (SCID) mice were obtained from
15 Dr. Leonard D. Shultz of the Jackson Laboratory, Bar
Harbor, ME. The mice were housed in standard isolator
cages within a routine animal holding facility. Under
these conditions, they have exhibited a life span that
was considerably shorter than that of inbred
20 immunocompetent strains (e.g., 1-2 years vs. 3-4 years).
The cause of death was normally related to opportunistic
infection (most often by *Pneumocystis carinii*). To
prevent infections, an antibiotic prophylaxis of
trimethoprim/sulfamethoxazole (40 mg/200 mg per 5 ml of
25 suspension; 0.125 ml of suspension per 4 ml of drinking
water per mouse per day) was administered. In all other
respects (e.g., bedding, food, daily light cycles, etc.),
the mice were handled as per routine animal holding
facility protocols.

Example 2

Collection and Preparation of Fetal Tissue

Tissue samples were obtained from human fetuses under about 24 weeks gestational age that were normal (absent any evidence of chromosomal defects, anencephaly,

-14-

hydrops, etc. as demonstrated by any available amniotic fluid analysis and/or ultrasonography data). Tissue samples were obtained directly in the operating room as fetal parts after an elective or medically-induced abortion. Without maintaining strict sterility, these parts were taken immediately to a gross dissection room. The fetal liver and thymus were identified, dissected out, and placed into RPMI 1640 medium with 10% fetal calf serum for transport to another lab. The fetal liver fragments were cut into sections of approximately 4 mm x 6 mm, to contain all of the representative cells of this organ (microenvironmental stromal cells, hematopoietic stem cells and their progeny, as well as hepatocytes). The fetal thymus fragments were cut into sections of approximately 2 mm x 2 mm. These tissues were normally introduced in as fresh a state as possible. Therefore, the tissue collection, preparation and implantation were done all on the same day. Implantation experiments using frozen tissues, however, worked well in the case of fetal liver cells and fetal thymus tissue. Therefore, aliquots of any remaining tissue were frozen in a 10% DMSO, 50% fetal bovine serum (FBS) in RPMI media, using standard procedures, and stored in a liquid nitrogen freezer.

25 Example 3

Transplantation of Human Tissue into SCID Mice

Generally SCID mice of 4-8 weeks of age were used for fetal tissue transplantation. The mice were anesthetized with ketamine, a 1 cm incision was made to expose the kidney, and the fetal liver and thymus tissue fragments were introduced, by means of a 19 gauge trocar, beneath the kidney capsule. The fragments were placed in close proximity so as to be in contact. Thereafter, the incision was approximated with surgical sutures. CB-17 SCID mice which received fetal human liver and thymus

-15-

tissue transplants, as described above, were designated SCID Thy/Liv mice. After the experiments described below were performed, the mice were dissected and it was found that they all had large thy-liv grafts approximately the same size as the kidney.

Example 4

Analysis of SCID-hu Thy/Liv Mouse Tissues

A. Immunophenotyping of Peripheral Blood Cells:

Peripheral blood samples were collected by tail vein incision or retroorbital bleeding, and approximately 100 μ l of whole blood was obtained, usually containing 1 to 2 x 10⁵ cells. Nucleated cells were enriched by precipitation of red blood cells with dextran sulfate, or by lysis of red blood cells with hypotonic shock, and washed free of platelets. Cells obtained from tissue samples of the thy-liv grafts, spleen and bone marrow were also analyzed. The cells were resuspended in fluorescent phycobiliprotein conjugates for analyses of cells and molecules (J. Cell Biol. ⁹³93:981-986), centrifuged at 200 xg, and the red blood cells lysed with hypotonic shock. The remaining cells were washed twice, incubated for 10 min with 1 mg/ml human γ -globulin (Gamimune, Miles Inc, Elkhart, IN) and stained as described below.

The cells were transferred to 96-well microtiter plates and stained with tri-color (TC) conjugated monoclonal antibodies to pan-human leukocyte marker CD45 (Caltag) and FITC-conjugated monoclonal antibodies to pan-human HLA class I marker W6/32 (derived from the W6/32 hybridoma obtained from ATCC). FITC conjugation are known in the art. Oi et al. (1982).

The staining was performed as follows: Cells were incubated in 10 μ l of staining buffer (PBS with 0.2 % bovine serum albumin (BSA) and 0.02 % sodium azide)

-16-

plus 30 $\mu\text{g/ml}$ FITC-conjugated W6/32 antibodies,
Tricolor-conjugated antibodies to CD45 diluted 1:45
(Caltag) and Ly5.1 purified from ascites were conjugated
to biotin succinimide ester and were used at 1-5 $\mu\text{g/ml}$.
5 Cells were incubated on ice for 20 minutes, followed by 2
washes with staining buffer. Cells were resuspended in
10 μl of staining buffer containing PE-conjugated
streptavidin diluted 1/40 (Becton Dickinson) and
incubated on ice for 20 minutes. Cells were washed twice
10 with staining buffer and resuspended in 300 μl of fresh
staining buffer for analysis. Stained samples were then
analyzed on a FACScan fluorescent cell analyzer (Becton
Dickinson) for cells positive for both W6/32 and CD45
after gating on cells with low forward and side scatter
15 properties. In this manner, the human cell content of
peripheral blood was determined as a function of total
mononuclear cells. ~~##~~ Blocks of fresh splenic tissue
were frozen in liquid nitrogen and stored at -70°C .
Cryostat sections of 8-10 μm thickness were fixed in
20 acetone for 10 min. and air-dried for at least 30 min.
After washing in 0.01 M PBS (pH 7.4) the sections were
incubated with the anti-human CD45 monoclonal antibody
(clone CLB-T200) which had been conjugated to horse
radish peroxidase (HRP) using periodate according to the
25 method described by Boorsma and Streefkerk, (1979)
J. Immunol. Met., 30:245. After washing, the peroxidase
activity was determined with 3,3'-diaminobenzidine
tetrahydrochloride (DAB, Sigma) in 0.5 mg/ml Tris HCl
buffer (pH 7.6) containing 0.01% H_2O_2 . The sections were
30 stained for 10 min. at room temperature. Acid
phosphatase in the cryostat sections was determined
according to the method described by Eikelenboom (1978)
Cell Tissue Res., 195:445.

35

-17-

Example 5Analysis of Human PBL Half-Life in SCID-hu Thy/Liv Mice

Initial studies indicated that the spleen may play a role in the depletion of human PBLs in SCID-hu mice. Figure 5 shows the level of human PBLs in SCID-hu mice under varying conditions. The cells were measured as described in Example 4. Figure 5A shows the initial production of human PBLs in SCID-hu Thy/Liv mice at more than three months after transplantation of the thymus and liver fragments. Figure 5B depicts the rapid drop in concentration of human cells after the human grafts are removed. Figure 5C shows a transient increase in peripheral human cells upon removal of the spleen from three to six month old SCID-hu Thy/Liv mice.

The results indicate that spleen removal results in a transient increase of human cells in some of the mice but the increase is not sustained.

Example 6Effects of Cl_2 MDP Injection

In order to determine the effect of depletion of murine macrophages on the ability of human cells to survive and circulate *in vivo* in SCID mice, a titration experiment was performed.

Liposome-encapsulated Cl_2 MDP was the generous gift of Dr. Nico van Rooijen. It was prepared with phosphatidyl choline and cholesterol according to the method described by Delemarre et al. (1990) Immunobiol. 180:395-404; and Rooijen (1989) J. Immunol. Meth. 124:1-6. Briefly, 75 mg phosphatidyl choline and 11 mg cholesterol (Sigma) were dissolved in chloroform in a round bottom flask. After low vacuum rotary evaporation at 37°C the lipids were dispersed by gentle rotation in 10 ml PBS in which 1.89 g dichloromethylene diphosphonate (Boehringer Mannheim, FRG) was dissolved. The resulting

-18-

liposomes were washed twice at 100,000 xg for 30 min. to remove free, non-encapsulated Cl_2MDP . The liposomes were then suspended in 4 ml of PBS to yield a "100% stock." Cl_2MDP was a gift of Boehringer Mannheim GmbH, Mannheim, Germany. Phosphatidylcholine (Lipid EPC), for preparation of the liposomes was a gift of LIPOID KG, Ludwigshafen, German.

Groups of four, 12 week old SCID mice received one initial, primary, injection of 200 μl 100% stock or 200 μl of 100% stock diluted in PBS immediately prior to injection. Three subsequent injections of 200 μl were given every 5-7 days as shown in Table 1. Four days after the final injection all treated mice and one group of 4 control untreated mice received 5.6×10^6 Ficoll purified human PBLs. Two mice from each treatment group were then sacrificed 24 hours and 72 hours later for analysis.

Table 1

Liposome Concentration

20

<u>Treatment</u>			<u>Primary Injection</u>		<u>Subsequent Injections</u>
.1			100%		50%
2			50%		25%
3			50%		10%
4			25%		10%
Control			0		0

25

The results obtained are shown in Figures 1 and 2. Human cells were detectable at low levels in peripheral blood, spleen and bone marrow of non-treated SCID mice 24 hours after intravenous injection when assayed by FACS as described in Example 4 and demonstrated in Figure 1 (control, 24 hrs). The cells were completely devoid of detectable cells in these

35

-19-

tissues within 72 hours post-injection as demonstrated in Figure 1, control (72 hrs). In contrast, human cells were detectable at high levels under all titration conditions at both 24 and 72 hours in these tissues, as demonstrated in Figure 1 (treatment 2).

In order to determine cell distribution, at the time of sacrifice 1/2 of the spleen was removed, frozen in liquid nitrogen, and stored at -70°C. The effectiveness of macrophage depletion was assessed by enzyme histochemistry for acid phosphatase, and the influx and localization of human cells was determined by using an anti-CD45 antibody as described in Example 4. Evaluations of sections was described as follows for acid phosphatase: (-) red pulp depleted of macrophages; (+/-) severely depleted, but some macrophages detectable in red pulp; and (+) no depletion. Evaluation for CD45 staining was as follows: (-) no CD45⁺ cells visible; (+/-) few CD45⁺ cells in white pulp areas or not all white pulp areas populated; (+) clear presence of CD45⁺ cells in white pulp areas; and (++) many CD45⁺ cells in white pulp areas.

These results are summarized in Figure 2 and show that a great proportion of human cells reside in the spleen of liposome-encapsulated Cl₂MDP treated animals with levels as high as 12.3% 24 hours post-injection and 6.7% 72 hours post-injection (Figure 2A, mouse 2-1 and 2B, mouse 2-4 respectively) compared to control mice where maximum levels in the spleen are 0.37% 24 hours post-transplant (Figure 2A, mouse C-2) and are undetectable at 72 hours post-transplant. In Figure 2, the percent human cell value was calculated as the difference between the proportion of cells found positive for W6/32 and CD45 compared to cells in the same quadrant when stained with irrelevant isotype control

35

-20-

FITC-conjugated IgG1 (Becton Dickinson) and TC-conjugated IgG2A (Caltag).

Although spleen cell numbers were reduced in treatment 1 mice (2.5×10^5 and 5×10^5 for mice 1-3 and 1-4 respectively) compared to control animals (2.7×10^6 and 1.1×10^6 for mice C-3 and C-4 respectively) treatment 2 mouse spleen cell content was not greatly altered (7.5×10^5 and 1.1×10^6 for mice 2-3 and 2-4 respectively). This indicates clearly that the observed retention in the spleen is not a concentration effect due to decrease in murine cell numbers, but is a preferential accumulation and survival of human cells. Under these conditions, human cells are also capable of circulating in the peripheral blood and are detectable in the bone marrow for at least 72 hours.

Splenic cryostat sections stained with acid-phosphatase to detect macrophages show complete elimination of red pulp and marginal zone macrophages with maintenance of some white pulp macrophages in liposome treated animals when compared to controls (Figure 2, macrophages). Cells staining positive for CD45 are not detectable in cryostat sections of control animals (Figure 2, CD45), in liposome treated animals. However, accumulation of human cells is found restricted to the white pulp and marginal zones. The extent of elimination of acid phosphatase positive cells in the red pulp region correlates with accumulation of human cells in the white pulp region. This is particularly obvious in treatment 2 mice where red pulp macrophages are completely eliminated, and the concentration of white pulp localized CD45⁺ cells is the highest. The proportion of human cells in the peripheral blood and the spleen is dependent on the treatment provided. The higher dose treatment regimens (Table 1, treatments 1 and 2) allow human cells to circulate in the peripheral

-21-

blood for at least 72 hours (Figure 2 mouse 1-3, 1-4,
2-3, 2-4). The observed effect however is most striking
in treatment 2 which is the optimum treatment regimen of
those tested for retention of human cells in peripheral
5 blood, spleen and bone marrow.

Example 7

Effects of a Single CL₂MDP Dose on the Number of Human Blood Cells Invading Peripheral Tissues

10 In order to determine if the human cells
protected by CL₂MDP treatment were circulating and
invading peripheral tissues, SCID-hu Thy/Liv mice were
injected in the tail vein with either 200 μ l of the
liposome-encapsulated CL₂MDP (N = 2) or 200 μ l of PBS
15 (N = 2). On day 4, blood samples were taken for analysis
of human cell content by immunophenotyping, as described
in Example 4. In addition, the Thy-Liv implants and
spleen were removed to quantify human cell content by
histological analysis, also as described in Example 6.

20 The Thy-Liv grafts from both liposome-
encapsulated CL₂MDP-treated mice and from PBS control
mice were essentially all (>99%) human and predominately
(approximately 70%) CD4⁺/CD8⁺ double positive. While the
two PBS control mice and one of the liposome-encapsulated
25 CL₂MDP-treated mice demonstrated low levels (0.5 to 1.0%)
of human cells in the spleen, the other liposome-
encapsulated CL₂MDP-treated mouse demonstrated a 5- to
10-fold increase in human cell content in the spleen (5%)
as compared with the human cell count observed from the
30 blood samples before and after the liposome-encapsulated
CL₂MDP injection (Figure 2).

35

-22-

Example 8The Effects of Cl_2 MDP on the Human Cell Content of
Peripheral Blood in SCID-hu Thy/Liv mice

In order to determine if a cellular constituent
5 of the spleen is responsible for depletion of human
cells, experiments were performed to assess the effect of
eliminating endogenous mouse macrophages by treatment of
the mice with liposome-encapsulated Cl_2 MDP.

Although the SCID-hu Thy/Liv mice have
10 substantial numbers of human cells in the grafts, the
numbers of circulating cells in the periphery remain low.
To test whether macrophages play a role in this
depletion, SCID-hu Thy/Liv mice transplanted one year
previously with fetal liver and thymus were screened for
15 peripheral blood human cell content at days 1 and 12. On
day 16, 200 μ l of either PBS or liposome-encapsulated
 Cl_2 MDP (100% stock) were injected into the tail vein of
these mice. Peripheral blood was then collected at
days 18, 21, 25, 32, 41 and 47 and analyzed by FACScan
20 for human cell content. Red blood cells were lysed, and
the remaining cells stained with W6/32-FITC, CD45-TC and
biotinylated mouse pan leukocyte marker monoclonal
antibody Ly5.1 with second stage PE-conjugated
streptavidin (Becton Dickinson). The percentage of human
25 cells was determined by cells double positive for W6/32
and CD45 and confirmed by being Ly5.1 negative. The
results are summarized in Figure 3.

Based on results observed for macrophage
recovery in normal (C3D2) F1 mice after liposome
30 treatment, if the decline in human PBLs were due to
murine macrophage, then an increase in human cells in the
periphery would occur immediately after injection,
followed by a return to baseline levels with the recovery
of murine macrophages within 1-2 weeks. Van Rooijen
35 et al. (1989). PBS control mice (N=3) did not

-23-

demonstrate a significant change in levels of human cells in the periphery following injection (Figure 3B). Five of 8 mice injected with liposomes, however, demonstrated a rise in human cells in the periphery 2-5 days post injection (days 18 and 21, Figure 3A), which was maintained at least 16 days post injection, and returned to base levels by 25 days post injection. The most striking of these were 2 mice in which human cell content in the peripheral blood rose from 0.3-0.5% pre-treatment to 2.8-3.2% post treatment (mouse 2 and 3, Figure 3A) and returned to 0.3-0.5% within 25 days post injection.

Example 9

The Effects of Cl₂MDP on Macrophage Content in the Spleen of SCID-hu Thy/Liv mice

A number of SCID-hu Thy/Liv mice were also treated with single and multiple injections of liposomes, and sacrificed to determine the efficiency of macrophage depletion in the spleen and correlate this effect with the proportion of human cells in the periphery. Macrophage content in the spleen was assessed by staining cryostat sections for acid phosphatase as described in Example 4. Human cell content was determined by both CD45 staining of cryostat sections and FACS analysis of splenic cells for co-expression of W6/32 and CD45.

SCID mice implanted with Thy/Liv grafts 11-12 months previously were given a treatment of 1 i.v. injection of 0.2 ml or 4 single i.v. injections every 5-7 days of either PBS or 100% stock and sacrificed for analysis 2, 3 or 8 days after the last injection (days after last injection). Prior to sacrifice a sample of tail vein peripheral blood (PB) was taken for flow cytometry. One-half of the spleen was homogenized and used for flow cytometry. Red blood cells were lysed, and cells stained with W6/32-FITC, CD45-TC and biotinylated

-24-

mouse pan leukocyte marker monoclonal antibody Ly5.1 with second stage PE-conjugated streptavidin. The percentage of human cells positive for both W6/32 and CD45 as determined by analysis on a FACScan is given (FACS W6/32-
5 CD45). One-half of the spleen was frozen in liquid nitrogen for cryostat sections and immunochemistry as described in Example 6. Murine macrophage and CD45 positive cell contents were also measured and evaluated as described in Example 6.

10 The results obtained are presented below in Table 2, where LIPS stands for liposome-encapsulated Cl_2 MDP and PB stands for peripheral blood.

Table 2

15	Mouse	Treatment (# of inj)	Days After Last Inj.	Spleen Immunochemistry		FACS	
				Murine Macs	CD45	W632-CD45 PB	Spleen
20	7732-4	PBS (1)	3	+	-	0.38	1.30
	7732-5	PBS (1)	3	+	-	0.31	0.40
	7732-1	LIPS (1)	3	-	+++	0.87	5.03
	7732-2	LIPS (1)	3	+		0.14	0.43
25	7634-4	PBS (4)	2	+	-	ND	0.14
	7634-5	PBS (4)	2	+	-	ND	0.01
	7636-5	PBS (4)	2	+	-	ND	0.28
	7541-5	LIPS (4)	2	+/-	-	ND	0.00
	7544-2	LIPS (4)	2	-	+++	ND	3.43
30	7636-3	LIPS (4)	2	-	-	ND	1.09
	7541-1	PBS (4)	8	+	-	0.70	0.03
	7544-5	PBS (4)	8	+	-	0.30	0.28
	7543-2	LIPS (4)	8	+/-	+/-	0.86	21.60
	7635-3	LIPS (4)	8	-	+++	0.81	30.69

-25-

As shown in Table 2, all PBS control mice maintained murine macrophage in the spleen, and had undetectable levels of CD45 staining in cryostat sections. Low levels of human cells were however detected in peripheral blood and spleen by FACS in control mice (up to 1.30%, Table 2, mouse 7732-4), suggesting the level of sensitivity for detection is higher by flow cytometry under these conditions. In all liposome-encapsulated Cl_2MDP treated animals where high levels of CD45^+ cells were detected in cryostat sections (Table 2, mouse 7732-1, 7544-2, 7635-3), disappearance of acid-phosphatase positive cells in the marginal zone and red pulp of the spleen was also noted.

The most striking examples of this were observed in mice that received 4 sequential injections and were analyzed 8 days after the last injection. Two control PBS mice (Table 2 mouse 7541-1, 7544-5) had low levels of human cells in the peripheral blood and spleen (less than 1.0%), and acid phosphatase positive cells in the spleen. However, the two mice that received liposome treatment demonstrated a large increase in human cell content observed by FACS with 30.69% for mouse 7635-3 in Table 2. Mouse 7635-3 had a 7-12 fold decrease in splenic cell number compared to the 2 control mice, suggesting again as for the PBL injections, that the result observed is due to a preferential accumulation and survival of human cells in the spleen.

Immunohistochemical and flow cytometry evaluation of macrophage depletion and human cell infiltration in liposome-encapsulated Cl_2MDP treated SCID-hu Thy/Liv mice were performed and the results are shown in Figure 4.

A) Cryostat sections of SCID-hu Thy/Liv mice treated with PBS (a and b) or Cl_2MDP liposomes (c and d) and analyzed for acid phosphatase (a and c) or stained with CD45 (b and d) as described in Example 4. wp: white pulp; rp:

-26-

red pulp of the spleen (magnification: 10x objective).
B) Flow cytometry analysis of splenic cells from mouse
7635-3, Table 2. Splenic cells were stained with W6/32-
FITC, CD45-TC and Ly5.1-Biotin plus SA-PE as described,
5 or with a combination of CD4-FITC. (Becton Dickinson),
CD8-PE (Becton Dickinson) and CD45-TC. Panel A: Forward
scatter versus side scatter showing the gated region
(R2). Panel B: Isotype control profile of IgG1-FITC and
IgG2a-TC staining. Panel C: Profile of cells stained
10 with W6/32-FITC and CD45-TC stained cells. Panel D:
Histogram of CD45 staining (solid line) and isotype
control (dashed line) with gates set for CD45 positive
cells (R1). Panel E: Histogram of CD8 positive cells
after gating on R1 and R2. The percentage of positive
15 cells is indicated in the top right corner. Panel F:
Histogram of CD4 positive cells after gating on R1 and
R2.

Staining of cryostat sections show very clearly the
effect of liposome mediated depletion on the spleen. In
20 control mice (Figure 4A, panel a) there is a uniform
distribution of acid phosphatase positive cells
throughout the white and red pulp area as well as the
marginal zone, and these areas are devoid of detectable
CD45 positive cells (Figure 4A, panel b). The liposome
25 treated mice show a near complete depletion of acid
phosphatase positive cells in the red pulp and marginal
zone (Figure 4A, panel c) and an accumulation of CD45
positive cells around the central arteriole of the white
pulp (Figure 4A, panel d). Further investigation of the
30 cell phenotype of mouse 7635-3 is shown in Figure 4B.
Cells gated for low side scatter (Figure 4B, panel A)
show the large percentage of W6/32-CD45 positive cells
(Figure 4B, panel C) compared to antibody controls
(Figure 4B, panel B). Cells that were gated for both low
35 side scatter and expression of CD45 (Figure 4B, panel D)

-27-

show a predominance of CD4⁺ cells (82%, Figure 4B,
panel F), the remainder being CD8⁺ (18%, Figure 4B,
panel E). These proportions (4:1, CD4:CD8) are within
the range observed in peripheral blood of most SCID-hu
5 Thy/Liv mice in our hands, indicating that the
infiltrating cell population consists of mature T-cells.

Although the foregoing invention has been described
in some detail by way of illustration and example for
purposes of clarity of understanding, it will be apparent
10 to those skilled in the art that certain changes and
modifications may be practiced. Therefore, the
description and examples should not be construed as
limiting the scope of the invention, which is delineated
by the appended claims.

15

20

25

30

35